Final Report

Original 2 of 2

Determination of the acute toxicity of [redacted]
against *Danio rerio* HAMILTON BUCHANAN
following EU-Method C.1 resp. OECD Guideline 203

Study No.: [redacted]

Sponsor:
[redacted]

Test Facility:
[redacted]

Monitor:
[redacted]

Study Director:
[redacted]

This page was intentionally left blank for statement of sponsor or submitter.

1 GLP-COMPLIANCE STATEMENT

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following guidelines:

- OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemikaliengesetz (Chemicals Act) of the Federal Republic of Germany (ChemG) § 19(a) to §19(d) and annexes 1 and 2, version 02 July 2008, Federal Law Gazette, Germany (BGBL) N. 28/2008, pp. 1146-1184, amended in Federal Law Gazette, Germany (BGBL) from 02 November 2011, N. 56/2011, pp. 2162-2169

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

I herewith accept responsibility for the data presented within this report.

There were no circumstances that may have affected the quality or integrity of the study.

The following parameters were not determined under GLP conditions:

- Analysis of drinking water, delivered by Verbandsgemeinde Maikammer
- Range-finding test (performed before preparation of the study plan):

[redacted]

[redacted]
Date

Information on Study Organisation:

Deputy Study Director	[redacted]
Study Plan dated	
Experimental Starting Date	
Experimental Completion Date	
Draft Report dated	

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice. Study Plan and Final Report were checked at the dates given below, the Study Director and the management were informed with the corresponding report.

Also, the performance of the study was inspected, and findings were reported to Study Director and management. The inspection of short-term studies (duration less than four weeks) is carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the reports were written in accordance with the Study Plan and the Standard Operating Procedures of the test facility.

Deviations from the Study Plan were acknowledged and assessed by the Study Director and included in the Final Report.

The reported results reflect the raw data of the study.

Verified Procedure	Inspected on	Findings reported on	Audit report no.
Study plan	[redacted]	[redacted]	
Performance of study			
Draft report			
Final report			

[redacted]

Date [redacted]

Quality Assurance Manager

Table of Contents

3 SUMMARY

Title of Study: Determination of the acute toxicity of [redacted] against *Danio rerio* HAMILTON BUCHANAN following EU-Method C.1 resp. OECD Guideline 203

Findings and Results:

The toxicity against *Danio rerio* was tested using a static test design.

The main study was performed as limit test with 100 mg/L nominal concentration. The treatment showed no mortality.

At the beginning and at the end of the test, the content of [redacted] component of the test item) in the test solution was analytically determined using AAS. Because of the [redacted] in test medium, the correlation between nominal and measured concentration was poor. Therefore, the determination of the results was based on the geometric mean of the measured concentrations.

The following results for the test item [redacted] could be determined:

96h NOEC ≥ 43 mg/L [redacted]
96h LC50 > 43 mg/L [redacted]

4 PURPOSE AND PRINCIPLE OF THE STUDY

This study was performed in order to evaluate the toxic potential of [redacted] towards freshwater fish, using the species *Danio rerio* HAMILTON BUCHANAN.

The fish were exposed to the test item for a period of 96 hours. Mortalities were recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 per cent of the fish (LC50) were determined.

Sponsor's intent: Notification in accordance with: REACH.

5 LITERATURE

The study was conducted in accordance with the following guidelines:

- OECD Guidelines for the Testing of Chemicals No. 203, adopted 17. Jul. 1992 "Fish, Acute Toxicity Test"
- Commission Regulation (EC) No. 440/2008, Method C.1: "Acute Toxicity for Fish" adopted 31. May 2008

Corresponding SOP of [redacted]

- [redacted]

6 MATERIALS AND METHODS

6.1 Test Item

Designation in Test Facility: [redacted]
Date of Receipt:
Condition at Receipt

6.1.1 Specification

The following information concerning identity and composition of the test item was provided by the sponsor.

Name [redacted]
Batch no.
Appearance
Composition

CAS No.
EINECS-No.
Molecular formula
Molecular weight
Purity
Homogeneity
Vapour pressure
Stability
Solubility
Production date
Expiry date
Storage
Hazard information
R-phrases
S-phrases

6.1.2 Storage

The test item was stored in a closed vessel dark and dry at room temperature.

6.1.3 Preparation

Because the solubility lies below 100 mg/L, the saturated solution was prepared for the test. This was done by weighing the nominal load, adding the corresponding amount of dilution water and shaking vigorously for 24 hours. The resulting solution was filtrated through 0.45 µm filters.

6.2 Test System

6.2.1 Specification

Species	*Danio rerio* HAMILTON-BUCHANAN
Age	sexually immature young fish, length 2 ± 1 cm

6.2.2 Origin

The original animals are obtained from Umweltbundesamt (UBA) and used for breeding and production of eggs.

The offspring of the original animals is used in the test.

6.2.3 Husbandry

Danio rerio is routinely used for toxicity tests. The test fish are kept following [redacted] in the current edition.

Vessels	polyethylene aquaria
Medium	chlorine-free tap water, full composition see annex
Feeding	three times a day with warmwater fishfood and daphnia, totalling to about 1-2% of body weight per day
Medium renewal	twice a week
Photo period	12/12 hours, using neon tubes
Temperature	23 ± 2 °C

6.3 Dilution Water

Good quality drinking water is used. The composition is analysed yearly by the supplier. The result of this analysis is given in the annex.

6.4 Instruments and Devices

The following instruments and devices were used for this study:

- Analytical Scales Mettler XS 205 DU[redacted]
- Automatic pipettes with one-way tips,[redacted]
- Glass measuring cylinders and flasks, 100 and 1000 ml
- pH-meter 340i wtw
- Oxygen meter Oxical 340i wtw
- Orbital Shaker GFL 3019[redacted]
- AAS contrAA 300 S

Usage and, if applicable, calibration of all instruments following the corresponding SOP in the current edition.

6.5 Analytical Method

The content of the test item [redacted] in the test solutions was determined by measurement of [redacted] as a component of the test item [redacted] using AAS. AAS measurements were performed with the flame technique (C_2H_2/air) at [redacted] nm for [redacted]

6.5.1 Sample Preparation

To each sample, 100 µL KCl (10% in water) and 100 µL HCl conc. were added and the mixture was filled up to 10 ml with test solution. Then [redacted] was measured in the C_2H_2/air flame at [redacted] nm. For the concentration 100 mg/L test item and the control, a 1:10 dilution was performed. 1000 µL test item solution was filled up to 10 mL with demineralised water after addition of 0.1 ml KCl (10% in water) and 0.1 mL HCl conc.

6.5.2 Parameters of Instrument

Specification: AAS contrAA 300, Analytik Jena AG
Absorption line [redacted]

6.5.3 Method Characterisation

Calibrated range: 0.01 – 2 mg/l [redacted]
Variation coefficient of method 3.28 % (calibration on 28.11.2011)
3.46 % (calibration on 02.12.2012)
Limit of detection [redacted] 0.01 mg/L [redacted]
Limit of quantification [redacted] 0.01 mg/L [redacted]
Note: the lowest calibration point is stated as limit of detection and quantification.

6.5.4 Accuracy

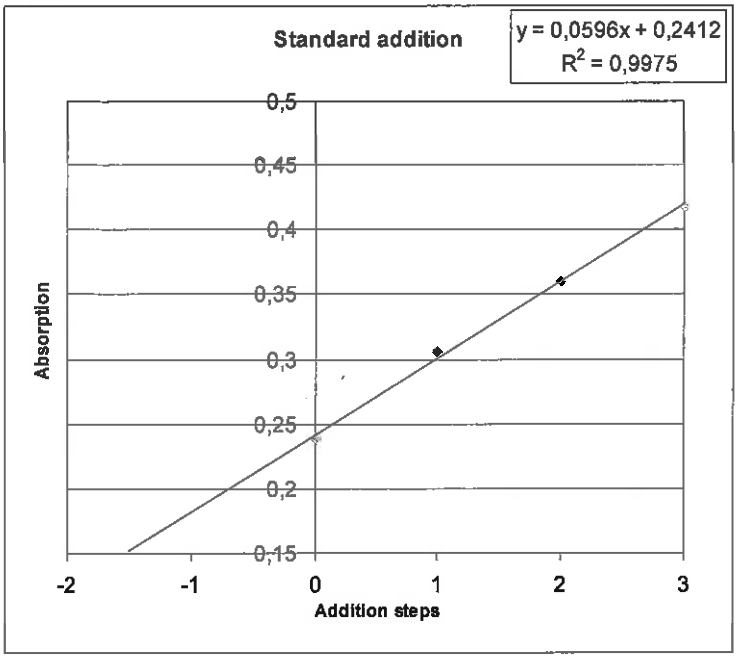
The accuracy in *Danio* medium was determined via standard addition. To 11.8 mg test item, 100 mL *Danio* test medium were added and the mixture was shaken for 24 hours at room temperature. After shaking, the turbid solution was membrane filtrated (0.45 µm), 5 mL of the filtrate was given in a 50 mL measured flask, 500 µL KCl (10% in water) and 500 µL HCl conc. were added and the flask was filled up to 50 mL with demineralised water (dilution 1:10).

Four 10 mL measuring flasks were prepared. Each flask was filled up to 10 mL with a diluted test item solution after addition of 0, 250 µL; 500 µL; 750 µL (respectively) of Zinc stock solution (10 mg/L). The samples were measured in triplicate.

Table 6.5-a Results Standard Addition Danio test medium

Addition	Amount of [redacted] stock sol.	Measured value (mean)	Measured conc. (mg[redacted]L)	Parameter of regression	Value
0	-	0.2376	0.572	Slope	0.059557
1	250 µl	0.3061	0.777	Intercept	0.241177
2	500 µl	0.36029	0.957	r	0.99873976
3	750 µl	0.41806	1.167	r^2	0.997481107

Graph of Standard Addition in *Danio* test medium:



6.5.5 Assessment

Conditions for the accuracy

Correlation coefficient r ≥ 0.996

The condition is fulfilled for *Danio* test medium with correlation coefficient r = 0.9987.

6.5.6 Stability of Solutions

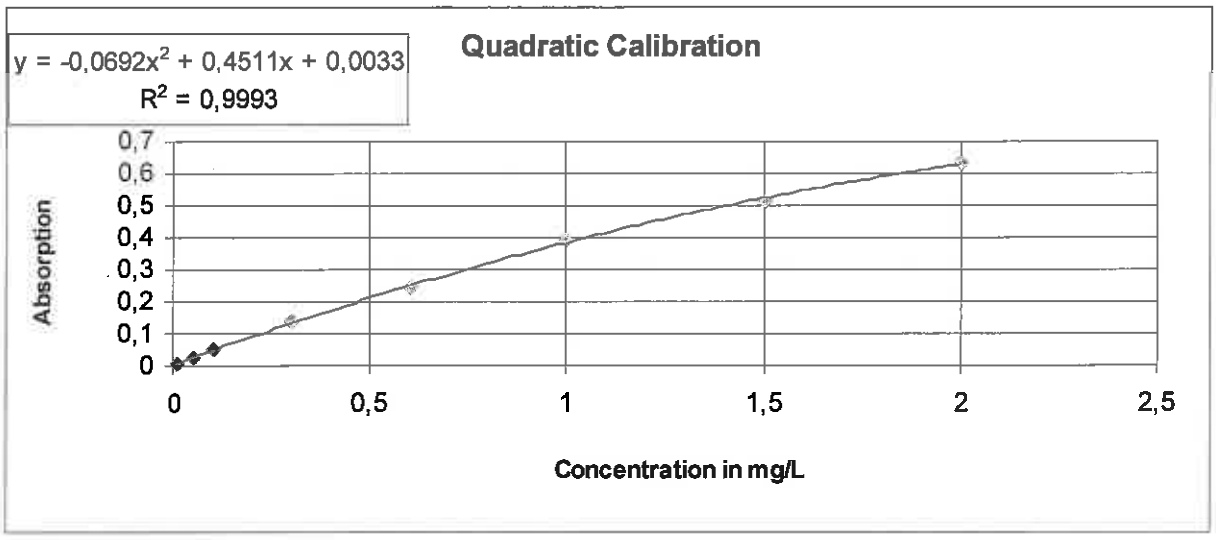
The determination of stability of the test item under the test conditions (aquaria, open, aerated) was determined with 200.1 mg Safire 400 in 2000 mL test medium in order to determine the concentration of the test item after 24, 48 and 144 hours. The data are presented in the following table.

Table 6.5-b Recovery Rates

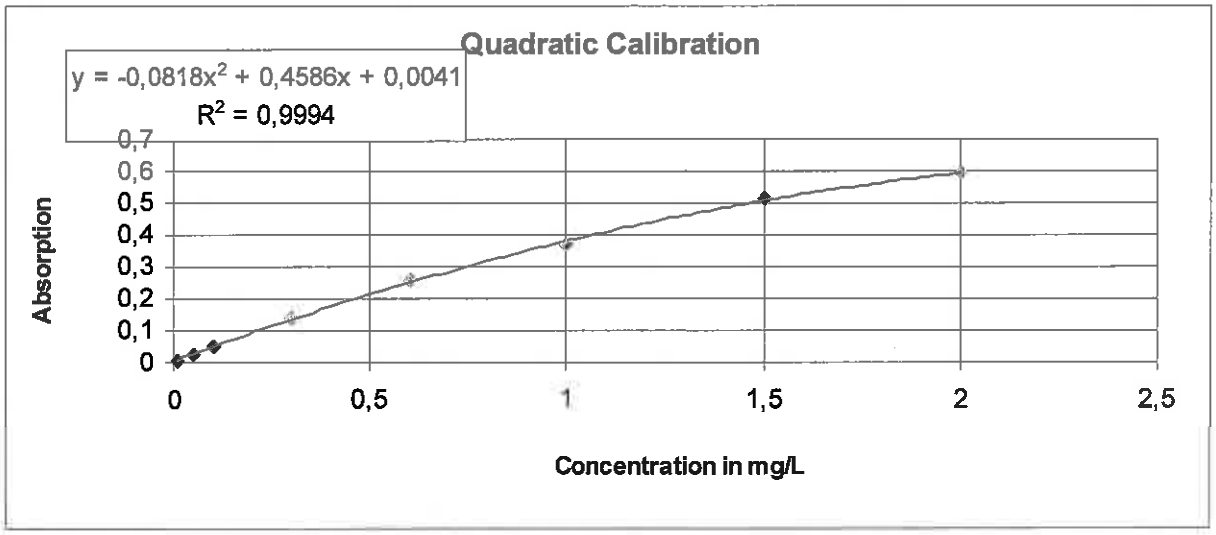
Time	Conc. [redacted]ean 1	Conc. [redacted]ean 2	Mean	Stability
0 h	0.501	0.486	0.494	100%
24 h	0.483	0.462	0.472	96%
48 h	0.468	0.462	0.465	94%
144 h	0.404	0.405	0.405	82%

6.5.7 Calibration Curve

6.5.7.1 Calibration of 28.11.2011



6.5.7.2 Calibration of 02.12.2011



7 CONDUCT OF THE STUDY

7.1 General Conditions

24 hours before the start of the test, food was withheld from the designated test fish.

Test Design	static
Medium renewal	none
Duration	96 hours
Loading	1 fish/L
Vessels:	glass aquaria, maximal volume 10 L
Aeration:	accomplished with glass tubes, frequency of bubbles 1/s
Feeding:	none
Photo period:	12/12 hours using neon tubes
Temperature:	24.2 – 26.2 °C
pH adjustment:	none
Replicates:	one vessel for each treatment and for the control

Observations were made every 24 hours, measuring pH and O_2-concentration of the test solution in each vessel and documenting mortalities or abnormal behaviour. The content of the test item in the test vessels was measured at the beginning and at the end of the main study.

A fish was considered dead, if no visible movement could be observed, and if touching of the caudal peduncle produced no reaction.

7.2 Main Study

On the day before the start of the study, the saturated solution was prepared for the test. This was done by weighing the nominal load, adding the corresponding amount of dilution water and shaking vigorously for 24 hours. The resulting solution was filtrated through 0.45 µm filters.

Date:	[redacted]
Treatment:	100 mg/L
Replicates:	one vessel, each containing 7 L test solution and 7 fish
Control:	one vessel, containing 7 L dilution water and 7 fish

8 FINDINGS

8.1 Main Study

8.1.1 Mortalities

The mortalities were recorded daily and can be found in the following table.

Table 8.1-a Mortalities

Nom. Conc. in mg/L	Total of fish used	dead fish			
		24 h	48 h	72 h	96 h
		cum	cum.	cum.	cum.
0	7	0	0	0	0
100	7	0	0	0	0

No abnormal behaviour of the fish was observed.

8.1.2 pH and O_2-Values, Temperature

The pH values in the test media and the control ranged from 7.0 to 7.8. The concentration of dissolved oxygen stayed above 7.5 mg/L or 87 % throughout the test. Temperature range was 24.2 – 26.2 °C. The details are given in the following tables:

Table 8.1-b pH values

Nom. Conc.	pH at t in h					O_2 in mg/L at t in h				
mg/L	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
0	7.7	7.7	7.8	7.7	7.6	11.4	8.8	7.8	8.8	7.8
100	7.0	7.5	7.4	7.5	7.4	12.1	8.6	7.5	7.6	7.5

Table 8.1-c Temperature

0 h	24 h	48 h	72 h	96 h
24.2	26.4	26.2	26.1	26.0

8.1.3 Analytical Determinations

The content of [redacted] of the test item) in the test solution was analytically determined using AAS. Because of the [redacted] in test medium, the correlation between nominal and measured concentration was poor. Therefore, the geometric mean of the measured concentrations was used for the determination of the results. Geometric mean is calculated by multiplication of the n participating concentrations and taking the n^{th} root

The details are given in the following table:

Table 8.1-d Measured Concentrations in mg/L and corresponding Recovery Rates in %

Nom. Conc. mg/L	Conc. [redacted] mg/L 0h	Conc. [redacted] mg/L 48h	Conc. Test Item mg/L 0h	Conc. Test Item mg/L 48h	% of Nom. Conc. 0h	% of Nom. Conc.48h	% Recovery	Geom. Mean. mg/L
0	< LOQ	< LOQ	--	--	--	--	--	--
100	5.96	5.32	45.0	40.2	45 %	40 %	89 %	42.5

LOQ = Limit of Quantification (0.01 mg/L [redacted]

8.2 Biological Results

The biological results are presented in the following table:

Table 8.2-a Biological Results

Parameter	Value	95%-confidence-interval
24h NOEC	≥ 43 mg/L	not determinable
24h LC50	> 43 mg/L	not determinable
24h LC100	> 43 mg/L	not determinable
48h NOEC	≥ 43 mg/L	not determinable
48h LC50	> 43 mg/L	not determinable
48h LC100	> 43 mg/L	not determinable
72h NOEC	≥ 43 mg/L	not determinable
72h LC50	> 43 mg/L	not determinable
72h LC100	> 43 mg/L	not determinable
96h NOEC	≥ 43 mg/L	not determinable
96h LC50	> 43 mg/L	not determinable
96h LC100	> 43 mg/L	not determinable

9 VALIDITY

- The mortality in the control may not exceed 1 fish at the end of the test.
  No mortalities occurred in the treatment and the control.
- The dissolved oxygen concentration must be at least 60% throughout the test.
  The concentration of dissolved oxygen stayed above 7.5 mg/L or 83 % throughout the test (100 % at 101300 Pa equals 8.57 mg/L, following DIN 38408 part 22).
- The pH-value in the test solutions should not vary more than 1 unit during the test.
  The highest variation was 0.5 units.

10 Discussion

All validity criteria were met.

As the test item is poorly soluble in water, a saturated solution was prepared. This was done by shaking the nominal load with the appropriate amount of dilution water for 24 hours, followed by membrane filtration. The main study was performed as limit test at 100 mg/L nominal concentration (limit of solubility). The treatment showed no mortality.

At the beginning and at the end of the test, the content of [redacted] component of the test item, [redacted] the test solution was analytically determined using AAS. Because of the [redacted] in test medium, the correlation between nominal and measured concentration was poor. Therefore, the determination of the results was based on the geometric mean of the measured concentrations.

No observations were made which might cause doubts concerning the validity of the study outcome.

The result of the test is considered valid.

11 Deviations

11.1 Deviations from the Study Plan

The following deviation from the study plan was documented:

- The temperature for the main study lay in a range of 24.2 to 26.4 °C and therefore outside the range which is stated in the guidelines. As no mortality occurred in the treatment and in the control, this deviation was stated as uncritical.

The deviation was signed and assessed by the study director on [redacted]

11.2 Deviations from the Guideline

See above.

12 Recording

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP Document Archive of the test facility for fifteen years. After that, the sponsor's instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP Substance Archive for fifteen years; then, the retain sample will be discarded.

Number of originals which will be sent to the sponsor: 1

13 ANNEX 1: COPY OF GLP-CERTIFICATE

RheinlandPfalz
LANDESAMT FÜR UMWELT, WASSERWIRTSCHAFT UND GEWERBEAUFSICHT

GUTE LABORPRAXIS - GOOD LABORATORY PRACTICE

GLP-BESCHEINIGUNG
STATEMENT OF GLP COMPLIANCE

gemäß/according to § 19b Abs. 1 Chemikaliengesetz

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

Prüfeinrichtung / Test facility

[redacted]

Prüfung nach Kategorien / Areas of Expertise
(gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6 , 8, 9 (toxikologische in Vitro Prüfungen an Säugerzellen und Bakterien)

Datum der Inspektion / Date of inspection
(Tag.Monat.Jahr / day.month.year)
29. und 30. November 2010

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist so rechtzeitig zu beantragen, dass die Folgeinspektion spätestens vier Jahre nach dem Beginn der o.g. Inspektion stattfinden kann. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit.

Verification of the compliance of the test facility with the Principles of the GLP has to be applied for in time to allow for a follow-up inspection to take place within four years after commencing the above mentioned inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Unterschrift, Datum / Signature, Date

[signature] 29.04.11

Dr.-Ing. Stefan Hill - Präsident -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht
Kaiser-Friedrich-Straße 7, 55116 Mainz
(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

MESSEN BEWERTEN BERATEN

14 ANNEX 2: ANALYSIS OF DRINKING WATER

Date of analysis: [redacted]

sodium	5.2	mg/L
potassium	2.6	mg/L
calcium	35.7	mg/L
magnesium	4.6	mg/L
aluminium	0.007	mg/L
iron	< 0.005	mg/L
mangane	< 0.005	mg/L
ammonium	< 0.02	mg/L
nitrate	8.5	mg/L
nitrite	< 0.01	mg/L
chloride	12.0	mg/L
sulphate	12.0	mg/L
total organic carbon (TOC)	0.5	mg/L
antimony	< 0.001	mg/L
arsenic	< 0.001	mg/L
lead	< 0.001	mg/L
cadmium	< 0.0002	mg/L
chromium	< 0.001	mg/L
copper	0.002	mg/L
nickel	< 0.002	mg/L
mercury	< 0.0001	mg/L
selenium	< 0.001	mg/L
bor	< 0.02	mg/L
cyanide	< 0.15	mg/L
fluoride	< 0.15	mg/L
benzene	< 0.25	µg/l
polycyclic aromatic hydrocarbons	< 4.0	µg/L
chlorinated organic compounds	< 1.0	µg/L
pesticides and biocides	< LOD	
pH	8.18	
conductivity at 20 °C	223	µS/cm
hardness	1.08	mmol/L

LOD = limit of detection